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Lipopolysaccharide Induced Apoptosis in Rat Hepatocytes

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Lipopolysaccharide Induced Apoptosis in Rat Hepatocytes

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ABSTRACT

The aim of this project was to determine if lipopolysaccharide (LPS) can induce apoptosis in primary cultures of rat hepatocytes and if this apoptosis is accompanied by modulation of Bcl-2 like proteins, or induced activation of the survival (Akt) or death [C-Jun-N terminal (JNK), p38 mitogen activated kinase (p38 MAPK)] kinases. The effect of LPS on glycochenodeoxycholate (GCDC) and Fas ligand (Fas L)-induced apoptosis was also investigated. The results of our study demonstrated that LPS induces apoptosis in rat hepatocytes and potentiates apoptosis in cells treated with GCDC or Fas L. No changes were seen in the expression of Bcl-2 proteins. However, a significant increase of p38 MAPK was seen in hepatocytes treated with LPS for 30 minutes.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

We would like to thank our Major Advisor, Dr. Cynthia Webster for allowing us to work in her lab, and for all her help throughout the entire project. Next, we would like to thank Anna Gates who showed us all of the specific techniques that we used to complete our project. Finally, thank you Dr. David Adams for helping initiate the project, and for help with the report writing.

BACKGROUND

The liver is one of the largest organs in the body and serves varied physiologic functions. The liver plays a role in carbohydrate, lipid and protein metabolism, detoxification of metabolites, storage of vitamins, trace metals, fat and glycogen, fat digestion, and immunoregulation. It also serves to filter and processes nutrient-rich blood arriving from the gastrointestinal tract. This ensures that the blood leaving the liver contains less waste than the blood that entered (Marieb and Hoehn, 2007). The liver is comprised of 4 basic cell types, hepatocytes, Kupffer cells (macrophages), sinusoidal endothelial cells, and fat storing stellate cells. The main functions of hepatocytes are fat and carbohydrate metabolism, storage and production of bile, as well as detoxification. Kupffer cells function in phagocytosis, antigen presentation, and secretion of proinflammatory mediators (Schoemaker and Moshage, 2004). Sinusoidal endothelial cells play a role in filtration, endocytic function, and regulation of sinusoidal blood flow (Shigeki & Imamura, 2000). Stellate cells are known to regulate retinoid homeostasis. However, these cells can also transform into myofibroblasts, which are the principal cellular source of collagen and other extracellular substances. Therefore, these cells play a central role in the pathological changes observed during the development of liver fibrosis (Senoo, 2004).

Hepatocyte Injury

Hepatocyte injury is caused by many different conditions. The most prevalent hepatic diseases in humans are viral hepatitis (hepatitis A, B and C), non-alcoholic steatohepatitis, alcoholism, and immune mediated disorders. Each of these diseases is known to create primary liver injury. In all of these conditions hepatocyte damage is associated with cell death (Schwabe

et. al., 2006). Cell death can be divided into two categories: necrosis and apoptosis. Necrosis is the result of metabolic disruption with energy depletion (loss of ATP), mitochondrial swelling, and activation of degradative enzymes. This causes cell lysis and expulsion of cell constituents into its surroundings. The presence of this intracellular debris stimulates an inflammatory reaction. Apoptosis is marked by DNA condensation, nuclear fragmentation, plasma membrane blebbing, and cell shrinkage. Eventually, the apoptotic cell breaks into apoptotic bodies, which are small membrane-surrounded fragments. These fragments are cleared by surrounding phagocytic cells. All of these apoptotic events are tightly controlled and well organized (Schoemaker and Moshage, 2004).

There are a number of secondary factors that can contribute to liver injury and thus contribute to hepatic failure (Figure 1). One important secondary liver injury results from absorption of lipopolysaccharide (LPS) or endotoxin from the gastrointestinal tract (Fig 1). Lipopolysaccharide is a glycolipid composed of a lipid portion, known as lipid A, as well as a sugar portion (Bauman, 2007). It is found on the outer membrane of gram-negative bacteria normally present in the intestine.

LPS contributes to liver injury by activating Kupffer cells. Kupffer cells are triggered by LPS to activate signal transduction pathways that promote the synthesis and release of cytokines and inflammatory molecules. When Kupffer cells are stimulated, chemostatic substances are released that attract circulating neutrophilic leukocytes. Stimulation also induces an up-regulation of adhesion molecules that attach to hepatic sinusoid cells that leads to neutrophil accumulation and activation in the liver. The activated neutrophils damage cells by the production of oxygen-derived free radicals. Kupffer cells also release the cytokine, tumor necrosis factor alpha (TNF-alpha) that can bind to hepatocytes and stimulate apoptosis. Direct

LPS binding to Kupffer cells or TNF-alpha binding to hepatocytes can also lead to pro-inflammatory signaling through activation of the transcription factor NK-κB, stress related kinases (JNK, p38 mitogen activated kinase), and interferon pathways (Schwabe et. al., 2006). Collectively these events lead to hepatocellular apoptosis and necrosis (Han, 2002).

Hepatocytes are also directly exposed to LPS as they are involved in the uptake of LPS from the hepatic sinusoids and its subsequent excretion into the bile. One previous study reported that LPS induces structural damage to hepatocellular mitochondria leading to ATP depletion and cell injury. It was also shown that LPS directly inserts into the lipid bilayer and activates signal transduction cascades that result in an increase in intracellular calcium, which induces hepatocyte necrosis (Han, 2002).

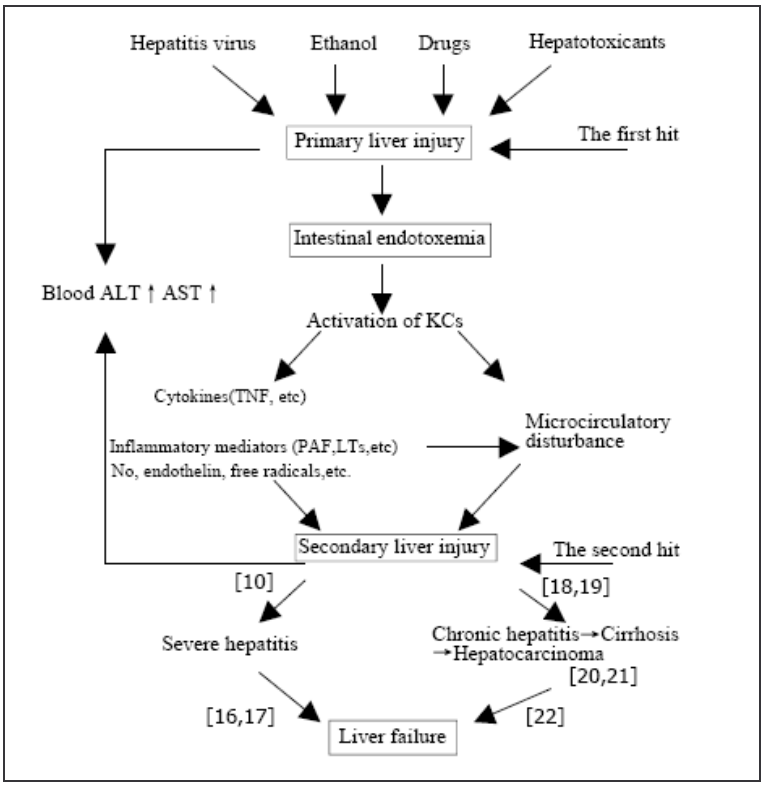


Figure 1: Diagram of Liver Failure. Primary liver injuries result in intestinal endotoxemia which activates cytokines and inflammatory molecules to induce secondary liver injuries. Secondary injuries can cause severe hepatitis then liver failure. Repeated secondary injuries can also cause chronic hepatitis and liver failure (Han, 2002).

Apoptosis

Apoptosis is a process in which cells that are no longer needed or injured are removed. Apoptosis can be triggered in a cell through two pathways, an extrinsic pathway and an intrinsic pathway. The extrinsic pathway, or death receptor pathway, is initiated by stimulating plasma membrane receptors, such as the Fas or TNF-alpha receptors. In contrast, the intrinsic pathway, or cellular stress pathway, is initiated by mitochondria or endoplasmic reticulum within the cell (Hague and Paraskeva, 2004).

In the extrinsic pathway (Figure 2), ligation of TNF-alpha or Fas family death receptors, by their natural ligands results in trimerization of the receptor. Once trimerization occurs, adapter molecules such as FADD (Fas Associated protein with Death Domain) associate with the death domain of the receptor that is on the inside of the cell. Procaspase-8 is recruited and the death inducing signal complex (DISC) is formed. Procaspase-8 is activated by autocleavage. In Type I cells, enough caspase 8 is cleaved to directly cleave and activate caspase 3. Caspase 3 is the final effector caspase and once activated apoptosis proceeds. In Type II cells, like hepatocytes, however, only a small amount of cleaved caspase 8 is generated and goes on to cleave BID. Caspase-8 cleavage of BID leads to translocation of the pro-apoptotic Bcl-2 proteins Bax and/or Bak to the mitochondria and subsequent disruption of the outer mitochondrial membrane with release of cytochrome C into the cytosol. An apoptosome, a complex of Apaf-1 (apoptotic protease activating factor 1), procaspase-9, and ATP, is formed. Caspase-9 is cleaved within this apoptosome and goes on to cleave and activate caspase-3 (Hague and Paraskeva, 2004).

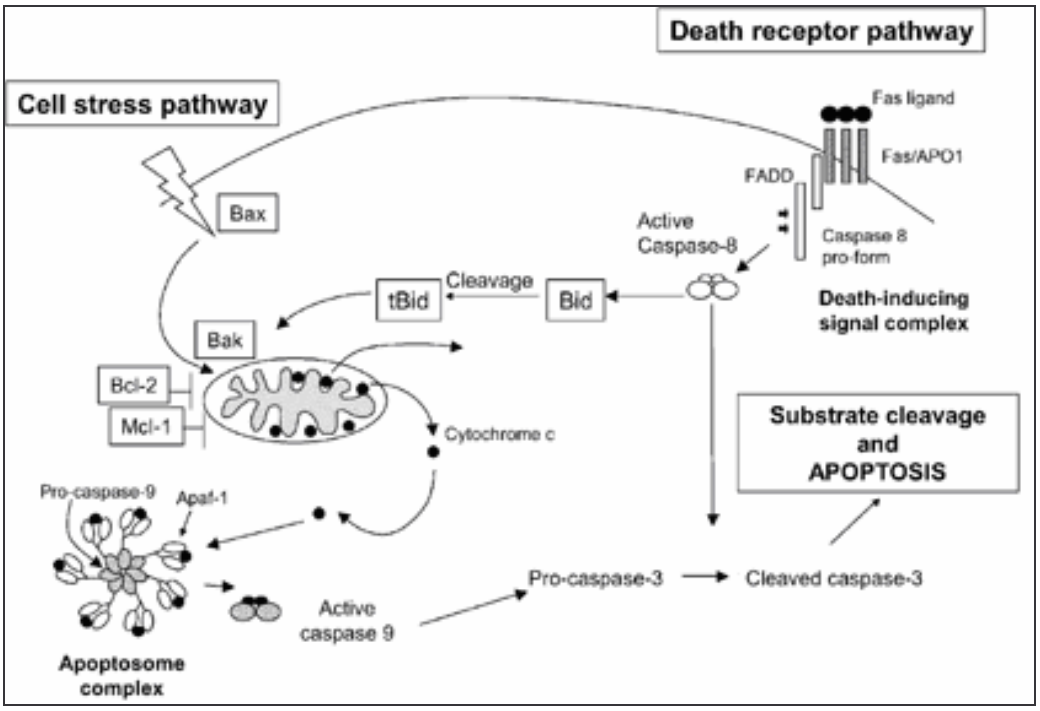


Figure 2: Apoptosis Pathways. The externally stimulated pathway induced by the Fas ligand is shown on the upper right. The internal pathway is shown centered on the mitochondria on the left. (Hague and Paraskeva, 2004).

Several members of the Bcl-2 family regulate apoptosis at the mitochondrial level (Fig. 3). At least 15 different Bcl-2 proteins have been identified and can be subdivided into pro-apoptotic and anti-apoptotic members. Molecules that are pro-apoptotic include Bid, Bad, Bim, Bax, and Bak, and molecules that are anti-apoptotic are Bcl-xL and Bcl-2 (Rust and Gores, 2000). Under normal conditions, anti-apoptotic proteins are predominately present in the outer mitochondrial membrane while pro-apoptotic proteins are located in the cytosol. Out of the Bcl-2 family proteins, Bak and/or Bax are necessary for causing apoptosis. When an apoptotic stimulus is recognized, Bax is translocated from the cytoplasm to the mitochondria, inserted into the membrane, and oligomerized. Bak is constitutively present in the mitochondrial membrane but held in check by the anti-apoptotic Bcl protein, Mcl-1. Both Bim and Bid can trigger activation of Bax and/or Bak. Once activated, Bax or Bak can then cause either a conformational change in

the mitochondrial membrane or a change in the mitochondrial permeability that allows cytochrome C to escape (Schoemaker and Moshage, 2004). Once cytochrome C is released apoptosis proceeds by sequential activation of the apoptosome and the effector caspase 3. Other Bcl-2 family members also regulate mitochondrial apoptosis. Bad, a proapoptotic protein is normally present in the cytosol, is kept in check by phosphorylation. Dephosphorylation and translocation to the mitochondrial membrane can lead to apoptosis by repression of Bcl-xL.

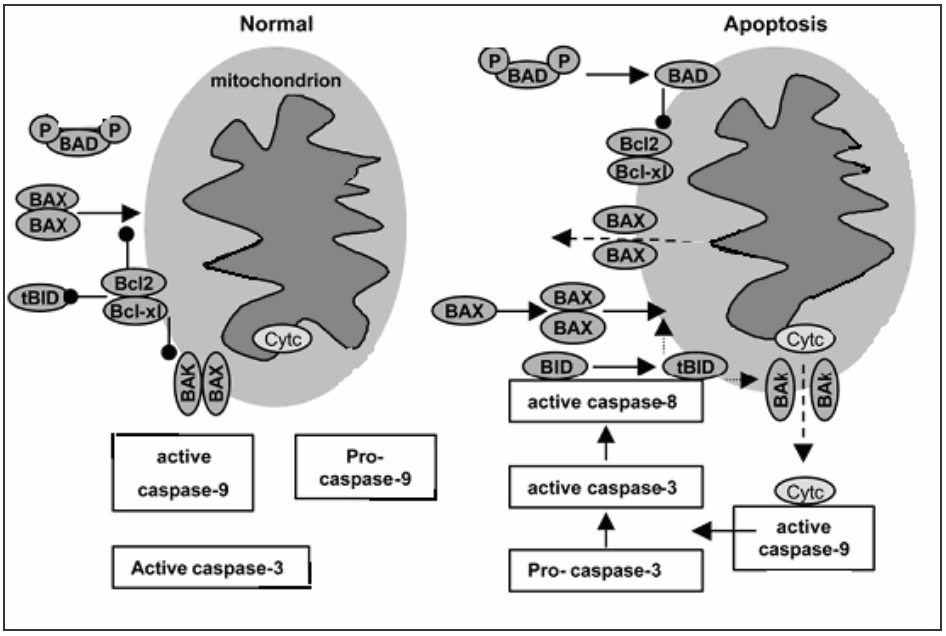


Figure 3: Mitochondrial mediated apoptosis. The mitochondria under normal conditions can be seen on the left panel. The mitochondria under apoptotic conditions can be seen on the right panel (adapted from Schoemaker and Moshage, 2004).

LPS signaling

Lipopolysaccaride binds to cell surface receptors known as toll-like receptors. Toll-like receptors (TLRs) are involved in the regulation of innate immune response. The TLRs mainly responsible for binding LPS are TLR4 and TLR2. When LPS binds to TLR4, pro-inflammatory

signaling is promoted through nuclear factor- κB (NK-κB), JNK, and p38 MAPK (Schwabe et. al., 2006).

Many types of cells in the liver contain TLRs such as stellate cells, biliary epithelial cells, sinusoidal endothelial cells, dendritic cells, Kupffer cells, and hepatocytes (Schwabe et. al., 2006). Kupffer cells and hepatocytes express TLR4 and TLR2, both of which are known receptors of LPS (Su, 2002). Therefore, both of these cell types are responsive towards LPS. TLR's transmit signals through four adapter molecules, MyD88, TRIF, TRAM, and TIRAP, which lead to pro-inflammatory, antibacterial, and antiviral signals. In Kupffer cells, LPS binding to the TLR4 receptors leads to the release of chemokines that lead to hepatocyte cell injury. When LPS binds to TLR4 receptors in hepatocytes, it may be taken up and removed from systemic circulation through secretion into the bile (Schwabe et. al., 2006).

Many *in vivo* studies in laboratory rodents have documented that LPS can cause hepatic injury. In some of these studies, LPS administration alone results in hepatocyte apoptotic cell death, while in others LPS alone does not damage hepatocytes but instead significantly potentiates the damage from known hepatotoxins (Barton et al., 2000; Kirsch et al., 2006). Still other studies have shown that virally infected, cholestatic, and cirrhotic livers have heightened sensitivity to LPS mediated damage (Jirillo et al., 2002; Moazzan et al., 2002; Tazi et al., 2007). In some cases, this enhanced sensitivity to injury has been associated with up-regulation of TLR4 expression in the liver (Mozer-Lisewska et al., 2005; Wang et al., 2005; Hua et al., 2007). It is unclear whether the damage to hepatocytes in these studies, which were done *in vivo*, is direct or is secondary to the release of inflammatory cytokines from LPS activated Kupffer and stellate cells. Very few studies so far have documented that administration of LPS to primary hepatocytes *in vitro* directly causes cell death (Yao et al., 2000; Han, 2002).

Bile acid induced apoptosis

Bile acids are a family of molecules synthesized from cholesterol exclusively in the liver. Bile acids are conjugated to either taurine or glycine and may have multiple hydroxyl groups attached (Hofmann et al, 1992). The most abundant bile acid in humans is the di-hydroxy bile acid, glycochenodeoxycholic acid (GCDC) (Webster & Anwer, 1998).

After bile acids are synthesized in the liver, they are secreted into bile and are stored in the gallbladder. Cholesytokinin (CCK) is released when fats are detected in the duodenum. When CCK is released, it contracts the gallbladder causing the release of bile acids. Bile acids form micelles in the duodenum which aid in the digestion and absorption of fats and fat-soluble vitamins. When the bile acids move to the ileum, they are reabsorbed by active transport and returned to the liver by the portal circulation. In addition to aiding in digestion and absorption of fat, bile acids also are the most potent stimulus for bile secretion and formation (Sherwood, 2004). The enterohepatic circulation system is 95% efficient and what is lost is replaced by the liver.

The hepatoxic potential of bile acids has been well documented. During cholestasis, when serum and hepatic bile acids concentrations increase due to decreased biliary clearance, bile acids damage hepatocytes. Studies have shown that toxic bile acids such as GCDC induce apoptosis as well as necrosis of hepatocytes depending on the concentration (Rust & Gores, 2000). At high concentrations, > 500 uM, bile acids form micelles and solubilize membranes causing cell necrosis. However, at lower concentrations such as 50-250 uM, bile acids cause apoptosis (Noto et al, 1998).

Bile acids induce apoptosis through a death receptor pathway. Bile acids trigger ligand independent oligomerization of cell surface death receptors, for example Fas and TRAIL (Sodeman et al, 2000; Grambihler et al., 2003). Bile acid-induced up regulation of Fas and TRAIL at the plasma membrane has been shown to involve activation of several protein kinases including protein kinase C, JNK and p38 MAPK (Jones et al, 1997; Gonzalez et al., 2000; Grambihler et al., 2003). Bile acids also have a direct effect on mitochondria to induce apoptosis (Figures 4 & 5). Bile acids may disturb the mitochondrial membrane leading to the mitochondrial permeability transition (MPT) (Figure 4). The MPT is the rapid increase of permeability in the mitochondrial membrane to solutes that would otherwise be prevented from passing through. Bile acids perturbation of mitochondrial membranes can lead to reactive oxygen species (ROS), which induce the MPT and the release of cytochrome c. Bile acids may also induce the translocation of cytosolic Bax to the mitochondrial membrane leading to pore formation and the release of cytochrome C.

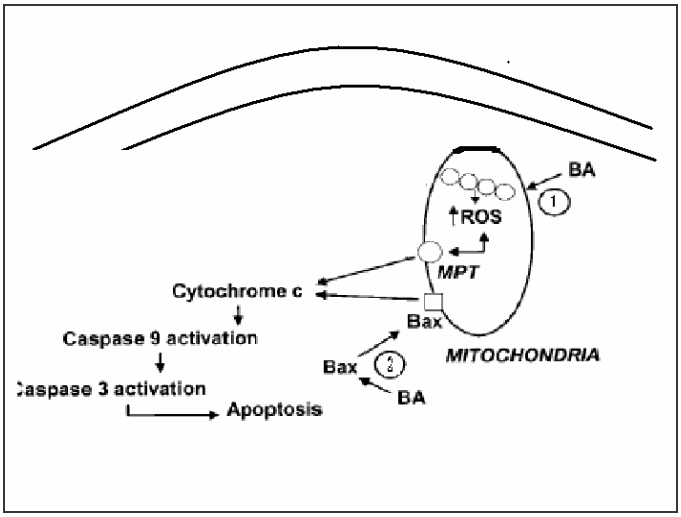


Figure 4: Proposed schema of bile acid (BA)-induced apoptosis. Bile acids may disturb the mitochondrial membrane leading to the mitochondrial permeability transition (adapted from Yerushalmi et al, 2001).

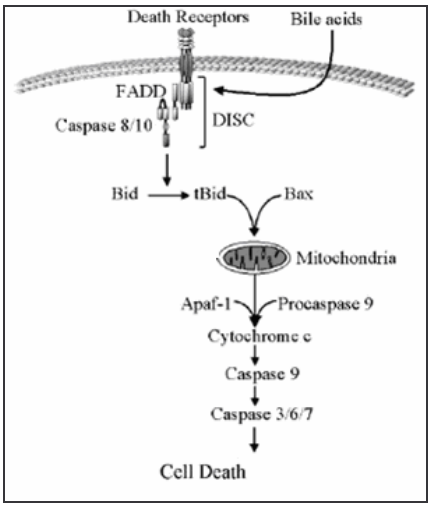


Figure 5: Bile acids promote death receptor mediated cell death signaling. The bile acid signaling pathway for apoptosis is shown including the caspase cascade (adapted from Higuchi and Gores, 2003).

In addition to promoting apoptosis, bile acids can also send anti-apoptotic signals. One of these signals is activation of the phosphatidylinositol 3-kinase (PI3K)/Akt system (Kennedy, 1997). Akt phosphorylates the pro-apoptotic protein BAD keeping it sequestered in the cytoplasm.

PROJECT PURPOSE

Although several *in vivo* studies have documented the detrimental effects of LPS on the liver it is unclear whether the damage is direct, or is secondary from the release of inflammatory cytokines from LPS-activated liver Kupffer and stellate cells. It is also unclear whether the hepatocyte apoptosis induced by bile acids (such as GCDC) or Fas ligand may be facilitated by LPS. The main objectives of this project were to determine whether LPS alone can cause apoptosis in primary cultures of rat hepatocytes, and whether LPS can potentiate the apoptosis induced by GCDC or a Fas ligand. Secondary objectives were to determine if LPS mediated apoptosis of rat hepatocytes was accompanied by modulation of Bcl-2 like proteins, and if LPS sensitization of GCDC-induced apoptosis was accompanied by alternations in GCDC induced kinase activation, specifically activation of the survival kinase (Akt) or death kinases (JNK or p38 MAPK).

MATERIALS AND METHODS

Cell Culture

Hepatocytes were isolated from rat livers by collagenase perfusion as previously described (Webster et al., 2002). Cells were plated on dishes or coverslips covered with Type I collagen in minimal essential media (MEM) supplemented with 10% fetal calf serum (FCS), 100 ng/ml of insulin, 100 ng/ml of penicillin, and 100 ng/ml of streptomycin. After 1 hour, media was changed to non-supplemented MEM for 3 hours. LPS (500 ng/ml) from *E*. coli (Sigma-Aldrich Chemical, St Louis, MO) was added at this time and allowed to incubate overnight. The next day cells were treated with either GCDC (Sigma-Aldrich Chemical, St Louis, MO) at 200 μM or Fas ligand (Axxora, LLC, San Diego, CA) at 50 ng/ml.

Monitoring of Apoptosis

Coverslips containing primary rat hepatocytes were processed 4 hours after the addition of 200 μM GCDC or 2 hours after the addition of 50 ng/ml of Fas ligand. Apoptosis was monitored morphologically using Hoechst staining. Apoptotic cells were identified by brightly staining condensed chromatin or nuclear fragmentation. A total of 500 cells were counted from random fields on each slide. Apoptosis was expressed as a percentage of the total number of cells counted. Biochemical confirmation of apoptosis was determined by immunoblotting cell lysates for the 17 kd proteolytic processing fragment associated with caspase 3 cleavage.

Preparation of Whole Cell Lysates

Whole cell hepatocyte lysates were prepared in cell lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton, 1 mM phenylmethlysulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 2.5 mM

sodium pyrophosphate, 1 mM β-glycerophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 500 nM okadaic acid, and 1 mM orthovanadate, pH 7.5) from cells either treated with 500 ng/ml of LPS overnight, 500 ng/ml LPS overnight and 200 µM GCDC for one hour, or treated with 500 ng/ml LPS for 5, 30, and 60 min. A Lowry assay was performed to determine the concentration of total protein (Lowry et al, 1951).

Immunoblots

Immunoblotting was performed to detect levels of Bcl-x_L, BIM, BAD, BAX, caspase 3, phospho JNK (Thr 183 & Tyr 185), phospho Akt (Ser 473), and phospho p38 MAPK (Thr180& Tyr182). Proteins were separated by SDS PAGE and transferred to a polyvinylidene fluoride membrane (Millipore) as previously described (Webster and Anwer, 1998). Information on the primary and secondary antibodies used is provided in Table 1. The blots were developed by chemiluminescence, digitized with Adobe Photoshop, and quantified using Sigma Gel software.

Antibodies	Company	Primary Antibody Solution	Secondary Antibody Used	Dilution of Primary Antibody
Bcl-xL	Cell Signaling Technology	5% NFM	anti-goat	1/500
Bim	Santa Cruz Biotechnology	5% NFM	anti-rabbit	1/1000
Bad	Cell Signaling	5% BSA	anti-rabbit	1/1000
Bax	Santa Cruz	5% NFM	anti-rabbit	1/1000
Caspase 3	Santa Cruz	5% NFM	anti-rabbit	1/1000
phospho-JNK (Thr 183 & Tyr 185)	Cell Signaling	5% BSA	anti-mouse	1/1000
phospho-Akt (Ser 473)	Cell Signaling	5% BSA	anti-rabbit	1/1000
phospho-p38 (Thr180& Tyr182)	Cell Signaling	5% BSA	anti-rabbit	1/1000
Total JNK	Cell Signaling	5% BSA	anti-rabbit	1/1000
Total Akt	Cell Signaling	5% BSA	anti-rabbit	1/2000
Total p38	Cell Signaling	5% BSA	anti-rabbit	1/1000
Actin	Calbiochem	5% NFM	anti-mouse	1/3000

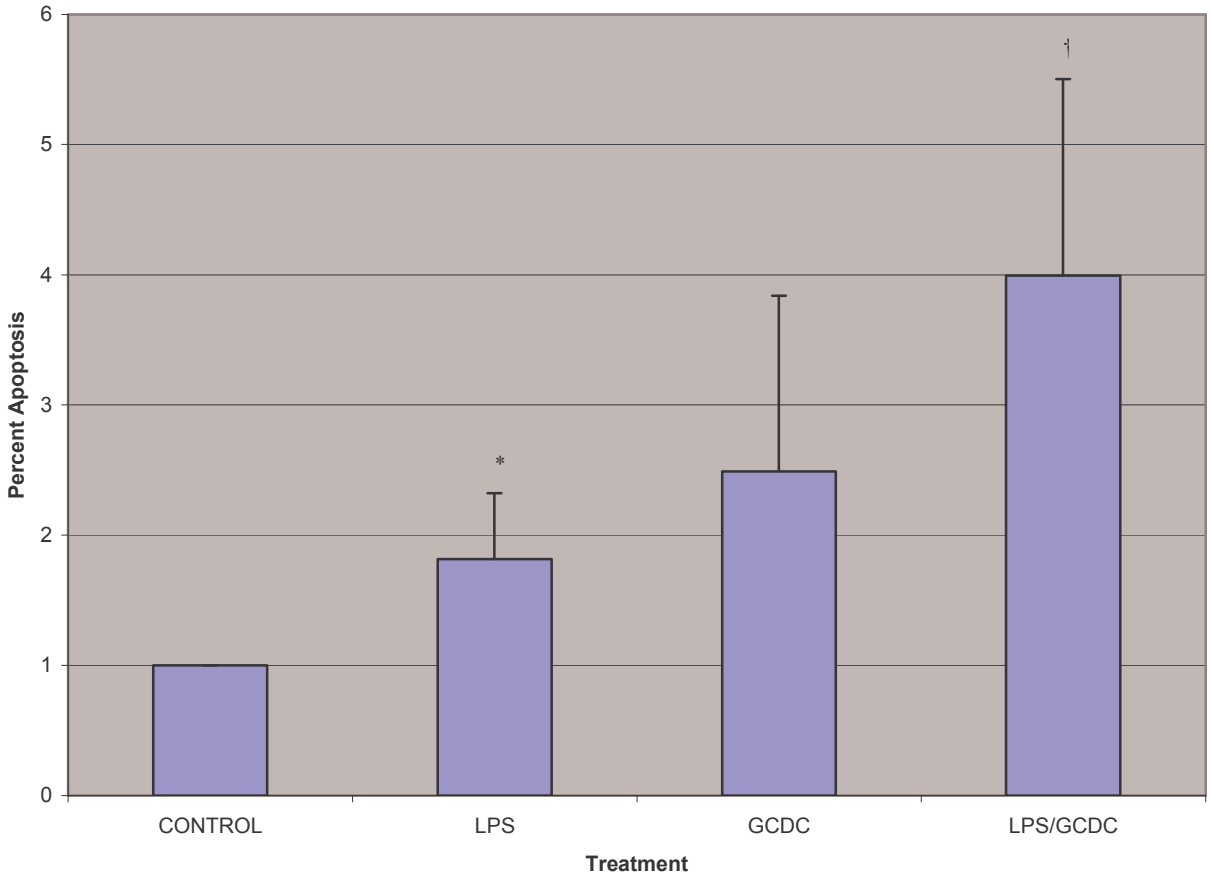
Table 1: Information on the antibodies used in this study
***NFM Non-fat milk, BSA- Bovine serum albumin; all secondary antibody was in 5% NFM.**

RESULTS

Potentiation of Apoptosis from LPS Treatment

Hoechst staining was used to identify the percent of apoptotic cultured primary rat hepatocyte cells treated with LPS, GCDC, or LPS + GCDC (Figure 6). Apoptotic cells were identified by their brightly stained condensed chromatin or by nuclear fragmentation. Overnight treatment of cultured primary rat hepatocytes with LPS (500 ng/ml) increased apoptosis an average of 1.8 fold relative to untreated cells (Fig 6 A, B). Treatment of hepatocytes for 4 hrs with 200 μM GCDC or 2 hours with 50 ng/ml of Fas increased apoptosis an average 2.5 fold, or 5.0 fold, respectively relative to untreated cultures (Fig 6 A, B). Overnight LPS treatment followed by 4 hr treatment with GCDC increased the amount of apoptosis 4.0 fold relative to untreated cultures (Fig 6A), while overnight treatment with LPS followed by 2 hr treatment with Fas ligand (Fig 6B) increased apoptosis an average 8.5 fold relative to untreated cultures. These data indicate that LPS can indeed directly increase hepatocyte apoptosis in the absence of potential signaling from Kupffer cells and their release of cytokines. The data also indicate that pre-treatment with LPS prior to treatment with known apoptotic inducers GCDC or Fas ligand potentiates the apoptosis.

A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4
3
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*
†
CONTROL
LPS
GCDC
LPS/GCDC
Treatment

B

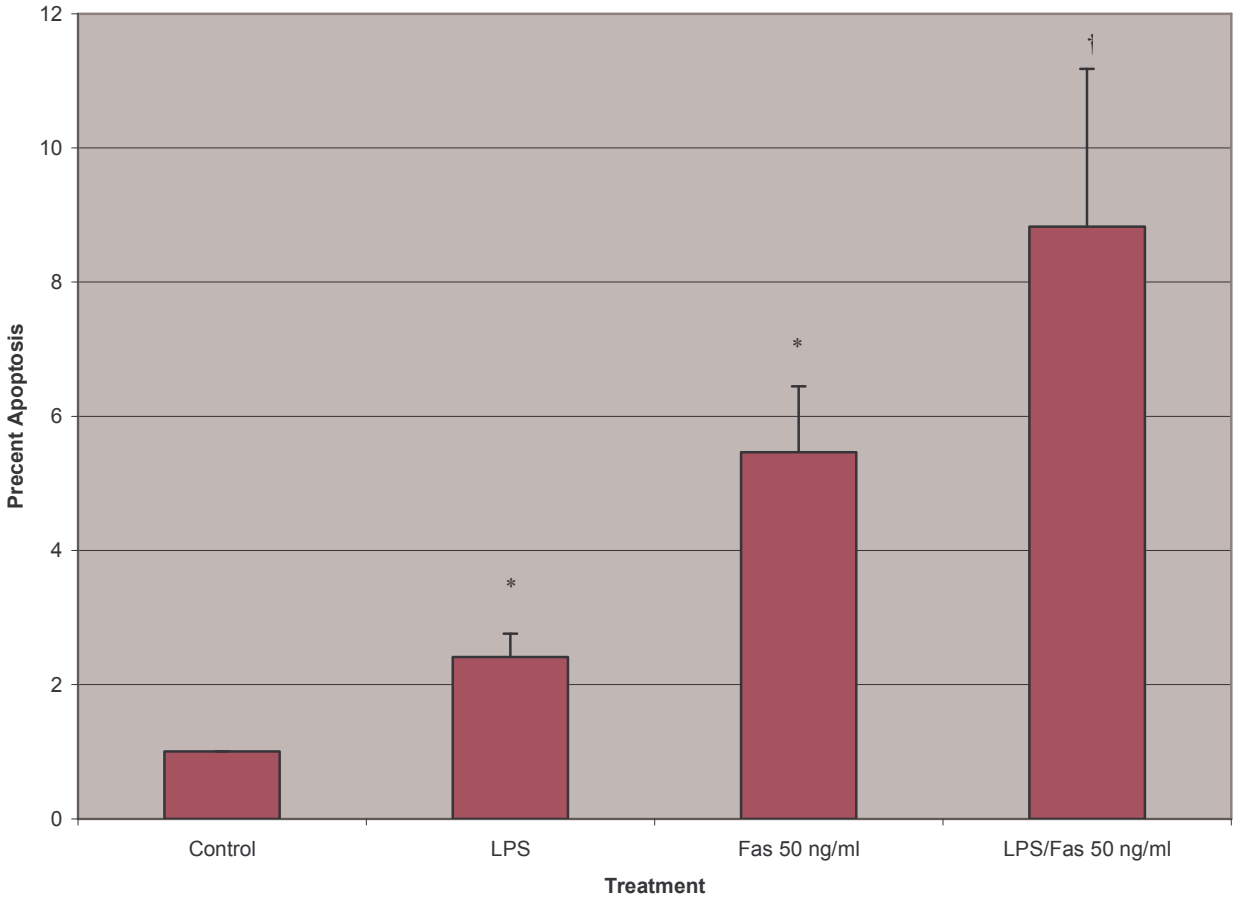


Figure 6: Effect of LPS on apoptosis in cultured primary rat hepatocytes. (A) Hepatocytes were treated with vehicle, 500 ng/ml LPS overnight, 200 µM GCDC for 4 hrs, or 500 ng/ml LPS overnight followed by treatment with 200 µM GCDC for 4 hours. Cells were evaluated morphologically by Hoeschst staining for the presence of apoptosis. Results are expressed as a percentage of the amount of apoptosis seen in the control and represent the mean +/- one standard deviation of at least 3 separate experiments (B) Experiment as in panel A except 2 hr Fas ligand treatment at 50 ng/ml replaced GCDC. * Significantly different from control. † Significantly different from LPS.

Role of Bcl-2 Family Proteins in LPS Induced Apoptosis

Several Bcl-2 family proteins have previously been shown to play a role in cellular apoptosis, so their potential role was investigated here. The role of the proapoptotic, Bax, Bad, and Bim, and anti-apoptotic Bcl-x_L Bcl-2 like proteins were investigated by immunoblotting cell lysates prepared from hepatocytes treated with 500 ng/ml LPS overnight. LPS had no statistically

significant effect on the Bcl-2 members tested (Fig 7). Equal loading in the immunoblots was verified with immunoblotting with actin (results not shown).

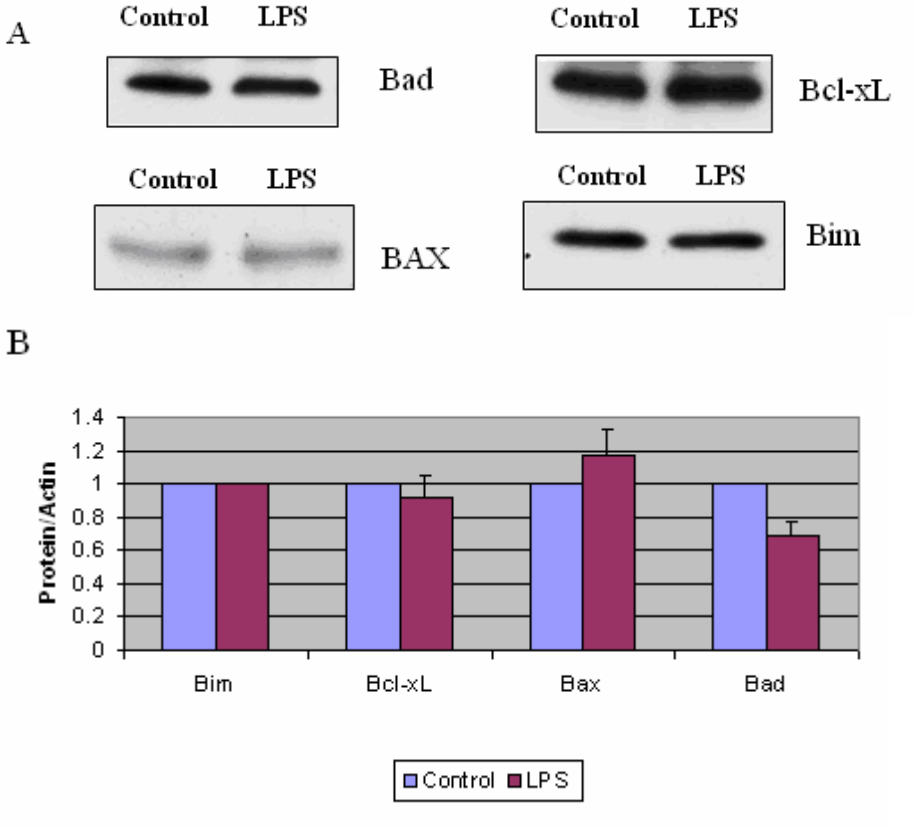


Figure 7: Immunoblots for various Bcl-2 family proteins. (A) Representative immunoblots. Cultured rat hepatocytes were treated overnight with 500 ng/ml LPS and the amount of Bad, BAX, Bcl-xL, and Bim determined by immunoblotting. The blots were developed by chemiluminescence, digitized with Adobe Photoshop and quantified. (B) Quantification of immunoblots. Bands present on the blots were quantified using Sigma Gel software. Each experiment was run twice, histobars represent the mean of two experiments, and error bars denote ± one standard deviation. The protein levels were corrected for any variation in protein loading by comparing to actin.

Role of Caspase 3 in LPS Induced Apoptosis

In order to look for biochemical indication of apoptosis, the cleavage of the main effector caspase, caspase 3, was investigated (Figure 8). Whole cell lysates were immunoblotted for the presence of the p17 cleavage fragment of caspase 3. Treatment with LPS plus GCDC (lane 3) or GCDC alone (lane 4) caused increased cleavage of caspase 3, but no cleavage could be detected

with LPS alone after overnight exposure (lane 2). The amount of p17 increased 2.0, 5.0, and 6.0 fold in LPS, LPS/GCDC, and GCDC treated hepatocytes, respectively.

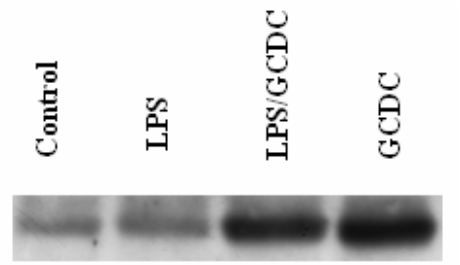
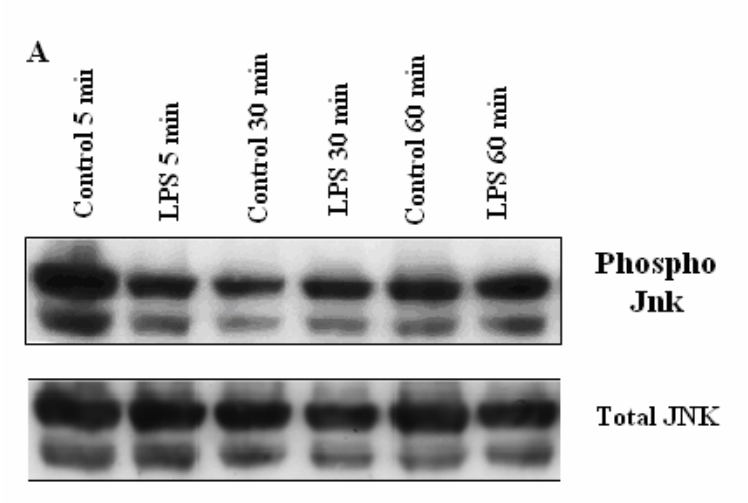


Figure 8: Immunoblot of 17 kD caspase 3 cleavage product. Cultured rat hepatocytes were treated overnight with 500 ng/ml LPS followed by 200 µM GCDC for four hours. Blots were probed for cleaved caspase 3. The blots were developed by chemiluminescence and digitized with Adobe Photoshop. Results are of n=1.

Activity of JNK, p38 MAPK, and Akt Kinases in LPS Induced Apoptosis

It has previously been shown that JNK, p38 MAPK, and Akt kinases are involved in TLR and bile acid signaling. Therefore, the activity of these three kinases was investigated in LPS and GCDC treated hepatocytes. A time course treatment with LPS was performed and the phosphorylation of JNK and p38 MAPK kinases was investigated (Fig 9). Although an increase in phospho-JNK was visible in LPS cultures relative to control after 30 minutes of treatment (Fig 9A), this increase was not statistically significant. For phospho-p38, a mean 2.8 fold increase was observed in LPS treated cultures relative to control at 30 minutes which was statistically significant (Fig 9B). This signal diminished to a 1.6 fold mean increase by 60 minutes which was not statistically different than the control.


A
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LPS 5 min
Control 30 min
LPS 30 min
Control 60 min
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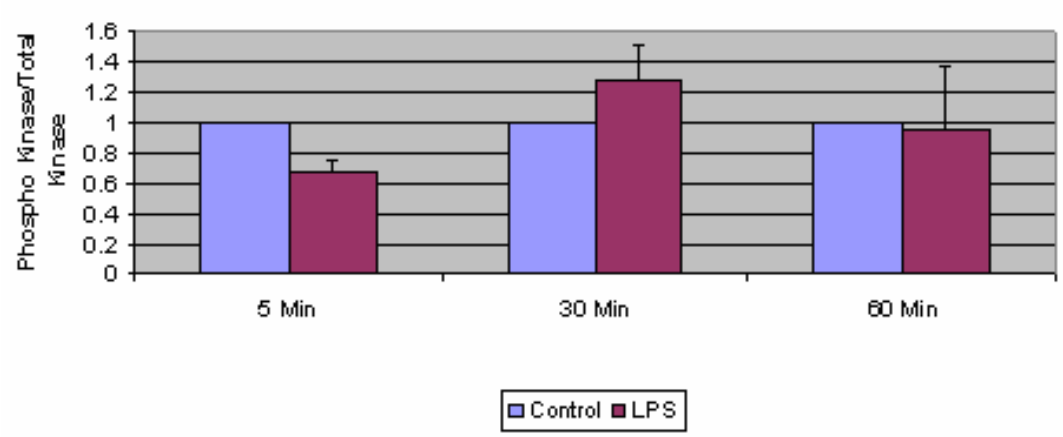
Total JNK


Phospho Kinase/Total Kinase
1.6
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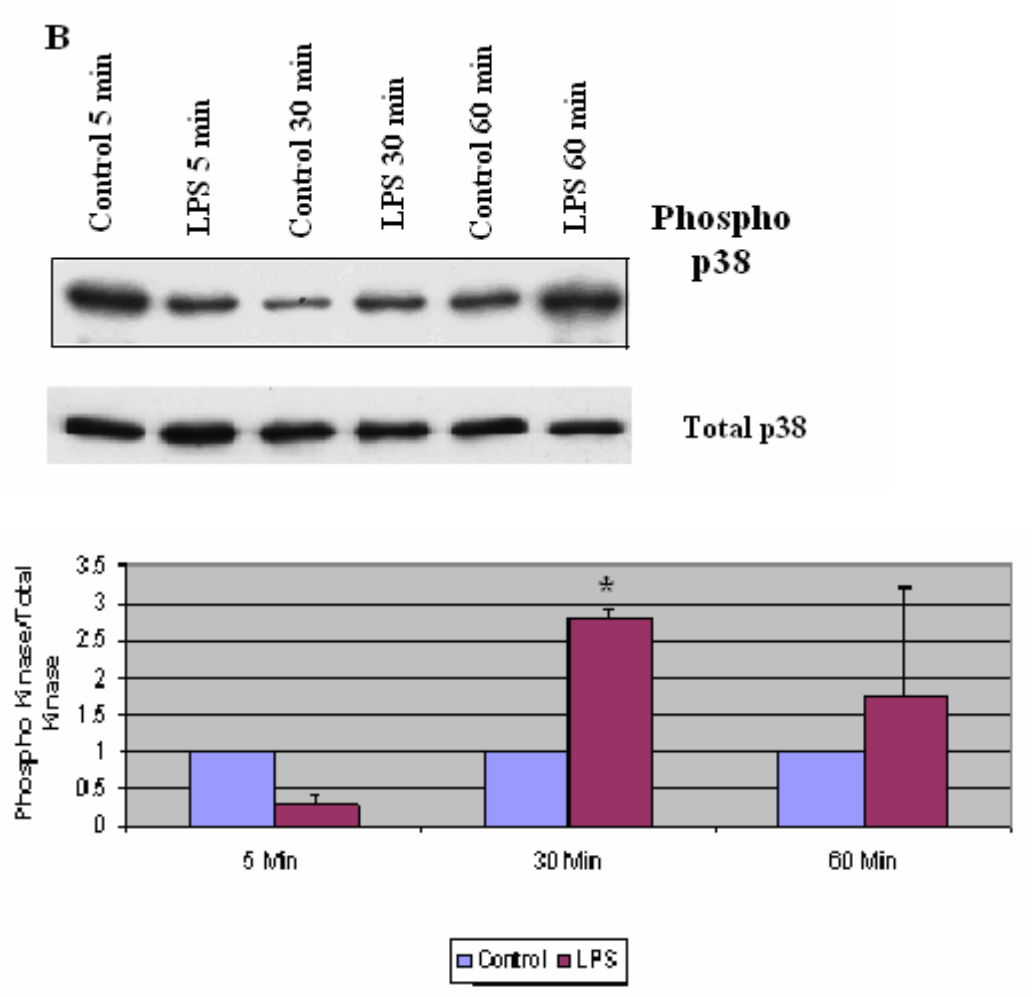
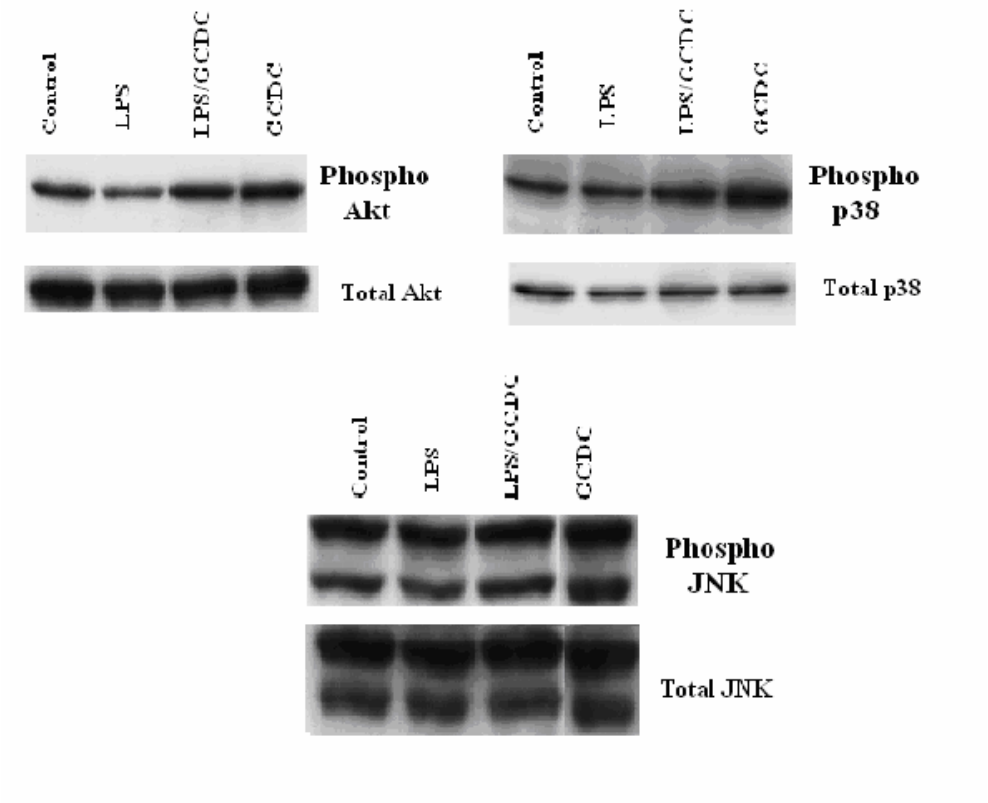


Figure 9: Effect of LPS on stress activated kinases. Cultured rat hepatocytes were treated with 500 ng/ml LPS for 5, 30, and 60 min and cell lysates were prepared. The lysates were immunoblotted for (A) phospho JNK and (B) p38 MAPK as well as respective totals. The blots were developed by chemiluminescence, digitized with Adobe Photoshop, and quantified with computer software. Representative immunblots are shown with the accompanying quantification. The results for JNK and p38 are the mean +/- SD of 2 experiments. The amount of phosphorylated kinase was corrected for any differences in the amount of total kinase. *Significantly different from control.

Although a statistically significant increase in p38 activity was observed following 30 minutes LPS treatment, we switched back to overnight treatments with LPS since our earlier experiments showed significant apoptosis with the latter treatments. The phosphorylation of JNK, p38 MAPK and Akt kinases was also investigated after overnight treatment with LPS and 4 hour treatment with GCDC (Figure 10). A 1.4 fold increase of p38 MAPK phosphorylation with

overnight LPS treatment was not statistically significant. There was also no significant change seen in the phosphorylation of Akt or JNK kinases with overnight LPS treatment. Treatment with GCDC resulted in a visible increase in Akt, JNK, and p38 MAPK (Fig 10B); however, these increases were not statistically significant. The combined treatment of LPS and GCDC shows that LPS actually decreases JNK phosphorylation compared to treatment with GCDC alone and had no effect on GCDC induced p38 MAPK or Akt phosphorylation.



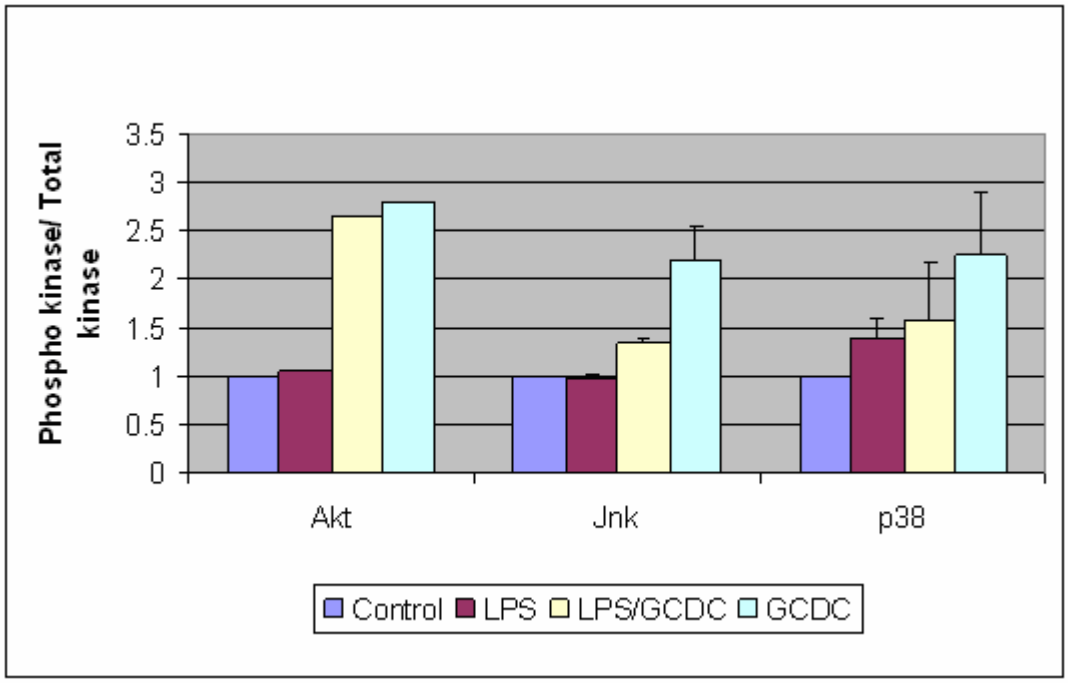


Figure 60: Effect of LPS on GCDC induced kinase phosphorylation. Cultured rat hepatocytes were treated over night with vehicle, 500 ng/ml LPS, 500 ng/ml LPS and then with 200 µM GCDC for 4 hours, or 200 uM GCDC for 4 hrs. Cell lysates were subjected to immunoblotting with antibodies for phospho Akt, phospho JNK, or phospho p38 MAPK. Immunoblots were developed by chemiluminescence, digitized with Adobe Photoshop and quantified with computer software. The amount of the phosphorylated kinase was corrected for any differences in amount of total kinase. The results of JNK and p38 are mean +/- SD of n=2 and the results of Akt are the result of n=1.

DISCUSSION

The present study shows that overnight treatment of rat hepatocytes with LPS alone causes hepatocyte apoptosis and potentiates the amount of apoptosis induced by GCDC or Fas ligand. Lipopolysaccharide modulation of apoptosis was accompanied by sustained activation of p38 MAPK kinase and was not associated with changes in the expression of Bcl-2 like family proteins BAX, BAD, BIM, and Bcl-X_L or modulation of the phosphorylation of JNK or Akt. Hepatocyte treatment with GCDC alone increased Akt, p38 MAPK, and JNK phosphorylation. Prior treatment with LPS had no effect on GCDC induced phosphorylation of p38 MAPK or Akt. However, LPS decreased GCDC induced JNK activation.

Our data show that treatment of cultured primary rat hepatocytes with LPS alone induces hepatocyte apoptosis. Although, it has been previously reported that LPS can induce apoptosis in cultured hepatocytes (Yao et al., 2000), that study failed to use morphologic criteria to confirm the cell death was actually apoptosis. Thus, our study is the first to show definitive morphologic evidence by Hoechst staining that LPS indeed causes hepatocyte apoptosis. This finding suggests that LPS can be directly responsible for causing liver injury without the activation of Kupffer cells.

Our data showed that LPS treatment of cultured rat hepatocytes results in increased phosphorylation of p38 MAPK. This might occur when LPS binds to TLR4 on hepatocytes. When LPS binds to TLR4, pro-inflammatory signaling is promoted through p38 MAPK and other pathways (Schwabe et. al., 2006). In a previous study, LPS was shown to have a direct effect on hepatocytes as increased phosphorylation of p42/p44 mitogen activated kinase was demonstrated (Liu et al., 2002). However, this study failed to show an increase in p38 MAPK at the same time points as the current study. This study might have failed to see p38 MAPK activity

due to a species difference because the previous study used mouse hepatocytes and the current study used rat hepatocytes. The current study shows that LPS can induce p38 MAPK phosphorylation in rat hepatocytes.

Previous investigations have shown that LPS induces hepatocyte apoptosis *in vivo*, but it is not well known how LPS induces apoptosis in hepatocytes. Studies have shown that LPS affects hepatocytes by binding to TLR's present on the cell surface. TLR's transmit signals through four adapter molecules, MyD88, TRIF, TRAM, and TIRAP, which lead to pro-inflammatory, antibacterial, and antiviral signals. In Kupffer cells, LPS binding to the TLR4 receptors leads to the release of chemokines that lead to hepatocyte cell injury. When LPS binds to TLR4, pro-inflammatory signaling is promoted through nuclear factor- κB (NF-κB), JNK, and p38 MAPK (Schwabe et. al., 2006). Some studies have demonstrated that p38 MAPK is pro-apoptotic in hepatocytes (Berra, 1998). However, there is controversy since other studies have shown that p38 activation is anti-apoptotic (Schoemaker et al., 2004). The role of LPS induction of p38 MAPK in hepatocyte apoptosis needs to be further clarified. Because our observed induction of hepatocyte apoptosis was achieved in the absence of Kupffer cells, our data supports a model in which LPS directly bound to hepatocyte TLRs induces apoptotic signaling.

The current study showed that there was no significant change in the Bcl-2 proteins Bim, Bad, Bax, or Bcl-xL after LPS treatment. However, during *in vivo* mouse studies it was shown that anti-apoptotic Bcl-xL, as well as pro-apoptotic Bad and Bax mRNA were upregulated by LPS treatment. The *in vivo* mouse studies on mRNA concentrations of Bcl-2 like proteins were performed at 4 hours, 8 hours and 12 hours after treatment. The current *in vitro* study on protein concentrations was performed at 5 minutes, 30 minutes and 1 hour (Zhong et al, 2006). Since, no up-regulation was seen in the protein levels for the Bcl-2 like proteins in the current study, it can

be suggested that either a species difference or time differential caused the disparity in results. However, it is also possible that if these proteins are involved in LPS induced apoptosis, it could be due to translocation of the proteins across the membrane or the phosphorylation state of the proteins, rather than the levels of the protein present that modulate apoptosis (Seminara et al, 2007).

Toxic bile acids such as GCDC and ligation of the Fas receptor induce apoptosis in hepatocytes (reviewed in: Rust & Gores, 2000). The current study concurs with this data. Also, the current study showed that treatment with LPS in conjunction with GCDC or Fas ligand potentiated hepatocyte apoptosis. There have already been ample *in vivo* studies showing that LPS can cause hepatic injury. In some of these studies, LPS administration alone results in hepatocyte apoptotic cell death, while other studies have shown that virally infected, cholestatic, and cirrhotic livers have heightened sensitivity to LPS mediated damage (Jirillo et al., 2002; Moazzan et al., 2002; Tazi et al., 2007). It can now be assumed that the LPS induced enhance hepatotoxicity may be due to the direct effect of LPS on hepatocytes.

Studies have shown that bile acid-induced apoptosis is associated with up regulation of Fas and TRAIL at the plasma membrane, and involves activation of several protein kinases including JNK and p38 kinases (Jones, 1997; Grambihler, 2003; Higuchi & Gores, 2003). The present study investigated GCDC-induced kinase phosphorylation in hepatocytes primed with LPS overnight. While GCDC induced activation of JNK, p38 MAPK, and Akt as expected, there was no significant effect of overnight LPS treatment on GCDC-induced p38 or Akt phosphorylation. Thus, LPS potentiation of GCDC-induced apoptosis cannot be explained by decreased bile acid induction of the pro-survival kinase, Akt or increase activation of the putative pro-apoptotic kinase, p38 MAPK. LPS did appear to decrease GCDC induced JNK activation.

Since GCDC induced JNK activation is thought to be pro-apoptotic, this effect of LPS can not explain the potentiation of apoptosis seen with LPS and would seem to be contradictory. However, the JNK's are actually a family of three kinases, and there is evidence that the different family members may have opposite effects in apoptosis (Barr and Bogoyevitch, 2001).

Our studies clearly demonstrate that LPS-induced apoptosis in hepatocytes and sensitized them to further apoptotic injury with toxic bile acids and Fas ligand. Future experiments will be needed in order to delineate the mechanism of LPS induced hepatocyte apoptosis. These experiments should include a determination of whether intrinsic (mitochondrial or ER) or extrinsic (death receptor) apoptosis pathways are necessary for the LPS induced effects. Caspases known to be associated with each apoptotic pathway could be assayed, such as caspase-9 for the mitochondrial intrinsic pathway, caspase-12 for the ER intrinsic pathway, or caspase-8 and caspase-10 for the extrinsic pathway. The initial activation time for each type of caspase could be assayed to determine whether an intrinsic pathway is activated secondarily to, or simultaneously with the extrinsic pathway. Thus, investigations into whether Fas or TRAIL death receptor expression or plasma membrane localization is altered with LPS exposure are warranted. It would also be beneficial to determine if Bid protein levels or subcellular localization are affected by LPS. Finally, the determination as to whether BAX translocates to the mitochondria during LPS apoptosis should be investigated. In addition, the role of TLR4 signaling in the action of LPS on hepatocytes could be further evaluated by determining if NF-κβ is activated in hepatocytes upon LPS stimulation and if this activation is necessary for LPS modulation of apoptosis. Since we have shown that p38 MAPK is stimulated by LPS, experiments using chemical inhibitors could be performed to determine if inhibiting p38 would prevent LPS induced apoptosis.

In conclusion, this is the first study to show that LPS alone induces hepatocyte apoptosis *in vitro* in the absence of Kupffer cells, and that it can potentiate apoptosis due to GCDC or Fas. These results show that LPS does not only cause liver injury independently but also increases injury when other pro-apoptotic factors are present. These results correlate with previous studies that have shown that LPS causes liver injury *in vivo*. Therefore, it can be determined that the direct apoptotic effects of LPS on hepatocytes may play a significant role in liver disease.

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